

Differing mechanisms of thrombin generation in live hematological and solid cancer cells determined by calibrated automated thrombography.

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Word Count 2994

Abstract 245

Figures 5

Tables 1

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21

22 Abstract

23 Objectives: Calibrated automated thrombography (CAT) is emerging as a reliable tool for real  
24 time estimation of thrombin generation (TG) potential. There is a clinical need for  
25 knowledge about the pathways underlying the thrombotic phenotype of different  
26 malignancies.

27 Methods: Cells from solid (e.g pancreatic cancer; n=7) and malignant hematological cell lines  
28 (e.g multiple myeloma; n=5) were evaluated for TG, using CAT, with the addition of control  
29 plasma (NormTrol) or plasma deficient in coagulation factors VII and XII. In addition, tissue  
30 factor (TF) cell surface expression was determined by flow cytometry.

31 Results: In platelet free plasma, TG in all cancer cell lines was cell concentration dependent,  
32 with the pancreatic cancer line CFPAC-1 producing the highest thrombin of 220nM at  $5 \times 10^6$   
33 cells/ml concentration. Lag times and times to peak reflected most significant differences  
34 out of all TG parameters measured and were inversely correlated with cell surface TF surface  
35 expression. Solid tumour cell lines had higher thrombin peaks, faster lag times, and a TG  
36 profile of overall greater magnitude than hematological cell lines. In the absence of Factor  
37 VII in platelet free plasma, TG in solid PC cell lines was significantly reduced unlike in  
38 hematological cell lines. However, in the absence of Factor XII, TG was reduced more in  
39 hematological cells but had little or no effect on solid cell lines.

40 Conclusion: The CAT assay identified characteristic differences in TG kinetics between solid  
41 tumour and hematological cancer cell lines, of which lag time and time to peak correlated  
42 with TF cell surface expression.

43

44	Key words:
45	Thrombin generation
46	Tissue factor
47	Calibrated automated thrombography
48	Venous thromboembolism
49	Coagulation factor-deficient plasma
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## Introduction

Thrombin is a key enzyme in hemostasis; it is a serine protease that forms the end product of the common pathway. The coagulation pathway is composed of intrinsic factors (e.g factor XII), and extrinsic factors such as tissue factor (TF) and factor VII complex that ultimately lead to thrombin generation (TG). Multiple myeloma (MM) and pancreatic cancer (PC) manifest a dysfunction of the hemostatic system that gives rise to a high incidence of thrombotic complications, particularly during systemic cancer treatment phase[1-3].

However, thrombosis may signify different things in both types of cancer. For example, it has been speculated that thrombosis in MM does not influence cancer behaviour and is mostly as a result of treatment, whereas in solid cancers such as PC, it is seen as a sign of aggressive tumour behaviour and poor prognosis indicator[4, 5]. Furthermore, molecular mechanisms involved in PC thrombosis development, such as TF expression, cyclo-oxygenase-2 (COX-2) and plasminogen activator inhibitor 1 (PAI-1) up-regulation, may ultimately not be important in MM-associated thrombosis due to its intra-vascular nature [4, 6].

To date few studies have been reported on TG variances using cancer cells themselves. There is limited knowledge on the thrombin generation kinetics in cancer (e.g measurement of thrombin output in various substrates, characterizing and profiling of thrombin generation, the influence of external pathways) and clinical and preclinical (*in vitro*) data in this setting are currently sparse.

Although several methods exist for assessing procoagulant activity, including tests for primary and secondary hemostatic function such as prothrombin time based clotting assays, fibrin formation to degradation and D-Dimer assays, and also newer approaches such as thromboelastography (TEG) and whole blood rotational thromboelastometry (ROTEM)[7], chromogenic assays are the cornerstone of measuring thrombin generation kinetics with the

calibrated automated thrombography (CAT) being a fluorogenic optimization that has made the leap from bench-side to clinical practice[8]. Technical optimization is ongoing to make it a point of care test. By giving a dynamic picture of thrombin generation through the measurement of the amount of thrombin cleaved from a fluorogenic substrate in real time, CAT may become a useful tool in cancer related clinical scenarios. Although it has been in existence in various forms since the 1950's, it was optimized by Hemker et al [9] into a dynamic modern version that measures 5 main parameters in TG: Lag time, Endogenous thrombin potential (ETP), Peak thrombin produced (Peak), time to peak (ttPeak or TTP), and Velocity index (Vel.Index). With this number of endpoints, the CAT assay gives a more dynamic clotting assessment and could play a role in clinical practice to assess clotting irregularities and abnormalities in patients with cancer related clotting disorders. However, more studies are needed to understand and address official standardisation issues for the assay results.

The traditional substrates for the CAT assay, as developed by Hemker et al, have been platelet poor and platelet-rich plasma. Few studies have documented the use of other substrates including whole blood[10, 11], tumour cells[12], lysed cells[13] *et cetera*. Although the components of the coagulation cascade are well characterised in hemostasis, there are limited experimental data directed at understanding their relative importance in the coagulopathy of solid versus non-solid malignancies. Factor VII and XII have distinct roles in thrombus formation [14] and understanding their contribution to the process of cancer associated thrombosis could inform clinical approaches to treatment and prophylaxis.

The aim of this study was to compare the characteristics of the thrombin curves generated by human solid malignant cell lines (with an emphasis on pancreatic cancer) versus the curves generated by hematological malignant cell lines (with an emphasis on myeloma cell lines), and to explore the role of the intrinsic and extrinsic pathways in TG. Therefore, we

103 investigated TG in an environment of factor VII and XII deficient human plasma. Tissue  
104 factor (TF) expression was also assessed by FACS in these cell lines, in order to gain an insight  
105 into its relationship with TG.

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## Methods

### Cell lines:

Solid tumour cells (pancreatic cancer AsPC-1, CFPAC-1, PANC-1, MIA PaCa-2, ovarian cancer SKOV-3, Head & Neck squamous cancer UMSCC81B and lung cancer PC9) and malignant hematological cell lines (Multiple myeloma MM.1S, U266B, H929, plasma cell leukaemia JJN3 and histiocytic lymphoma U937) were used in this study. All cell lines were purchased from the American Type Culture Collection (ATCC) or the European Collection of Authenticated Cell Cultures (ECACC) except UMSCC81B (Dr Thomas Carey) and JJN3 (Dr Guy Pratt). CFPAC-1, ASPC-1 and MIA-PaCa-2 cells were maintained in Iscove's Modified Dulbecco's Medium (ATCC), supplemented with 10% FBS (Bio-Sera, UK), 1% L-glutamine and for MIA-PaCa-2 cells only, 2.5% (v/v) horse serum from Gibco® (Life Technologies Ltd, UK). SKOV-3 was maintained in McCoy's 5A medium supplemented with 1% L-glutamine and 15% (v/v) FBS. Hematological cell lines were cultured in RPMI 1640 medium (ATCC) supplemented with 10% (v/v) FBS. All media were supplemented with 1% penicillin/streptomycin. Cell viability was assessed before each assay by trypan blue exclusion, and cells with  $\geq 90\%$  viability were used.

### Calibrated Automated Thrombogram assay (CAT):

TG was assessed by the Hemker et al method [9] using a calibrated automated assay in real time. Here, 20 $\mu$ l of live cancer cells at various cell concentrations were plated into Immunulon transparent round bottom 96-well microtiter plates (ThermoFischer Life Sciences, Loughbrough, UK) for the CAT assay. 20  $\mu$ l of PPP-Reagent LOW (Diagnostica Stago, Theale, UK) which contained 1pM recombinant relipidated TF and 4 $\mu$ M phospholipids standard preparations in HEPES-buffered saline was used as positive control, while 20 $\mu$ l of PBS was the negative control. 80 $\mu$ l of platelet-free normal control plasma (NormTrol) or plasma deficient in coagulation factors VII and XII were added to the microplate wells. All

plasmas were purchased as freeze-dried pooled immunodepleted human plasma (Helena Biosciences, Gateshead, UK). Then the microplates were agitated and incubated for 10 minutes while TG was initiated by addition of 20µl of a FluCa Kit solution (Diagnostica Stago, Theale, UK) containing 0.1M CaCl<sub>2</sub> and a fluorogenic substrate (Z-Gly-Gly-Arg-amino-methyl-coumarin fluorophore). The fluorescence emission was measured by Fluoroscanner Ascent® fluorometer (Thermolab systems OY, Helsinki, Finland) with excitation/emission wavelengths 390nm/460nm. Measurement occurred up to 1 hour after automatic mixing of contents of the wells at every 20 seconds, according to the Thrombogram guide [15]. Real time TG curves were calculated on a Thrombinoscope™ software version 3.0.0.29 (Thrombinoscope B.V., Maastricht, The Netherlands). Each condition contained 2 wells, one test well for cell induced TG, the other a calibration well as control for thrombin activity in test well. 20µl of Thrombin Calibrator α<sub>2</sub>-macroglobulin/thrombin complex (Thrombinoscope B.V., Maastricht, The Netherlands) was used. Each experiment was carried out in triplicate. Time to start of TG (Lag time), endogenous thrombin potential (ETP), Peak height of thrombin (Peak), time to peak (TTP or ttPeak) and velocity index (Vel.Index) of TG were evaluated.

#### Flow cytometry:

TF cell surface expression was measured by incubation of 50µl of cells at 1x10<sup>6</sup>/ml per tube from each cancer cell line with 5µg of anti-human CD142: FITC antibody or an isotype-matched negative control (IgG<sub>1</sub>; Bio-rad, UK). This mixture was left in the dark for 30 minutes at room temperature, washed twice with PBS, and then resuspended in 300µl of PBS before flow cytometry on a BD FACSCalibur running CELLQUEST PRO software (BD Biosciences, Oxford, UK). Forward scatter (FSC) and side scatter (SSC) were set at logarithmic gain and the mean fluorescence ratio (CD142/negative control) for each sample was evaluated and repeated three times on different days.



Prothrombin time:

At decreasing cell concentrations ( $5 \times 10^6$  -  $4 \times 10^4$  cells/mL), UMSCC81B suspended in PBS (100µl per curvette) were incubated with 100µl of NormTrol plasma (Helena Biosciences, Gateshead, UK) for 4 minutes at 37°C in a Thrombotrack SOLO coagulometer (Alere, Stockport, UK) as previously described [16]. The clotting reaction measures the time taken for fibrin formation in plasma and was initiated with 100µl of  $\text{CaCl}_2$  at 0.025M concentration, with results as average of measurements repeated on three separate occasions  $n=3$ .

Statistical Analysis:

The results are mean  $\pm$  standard deviation. Student t-tests and Analysis of variance (ANOVA with multiple comparisons) were used to determine statistical significant differences between groups. P values less than \*0.05, \*\*0.01, \*\*\*0.001 were considered significant. Relationships between TG parameters (Lag time, ETP, Peak, TTP, Vel.Index) and TF cell surface expression was assessed with Pearson correlation coefficient on linear regression analysis.

## Results

### *Thrombin generation in platelet free plasma*

Initially, 12 different cell concentrations were used to identify the optimal concentration at which TG signal is detectable in both solid and hematological cancers by the CAT assay. TG was assessed in decreasing cell concentrations from ( $5 \times 10^6$  -  $4 \times 10^4$  cells/mL) and was concentration dependent in both cancer types (Figure 1). Although ETP in both solid and hematological cells remained similar, there were linear decreases in Lag times and times-to-peak such that as concentration decreases both increased in duration. The inverse was seen for peak thrombin and Vel. indices. Furthermore, hematological cells had overall smaller TG curves compared to the solid cancer cells. UMSCC81B, CFPAC-1 and AsPC-1 cell lines initiated TG quickest within  $1.5 \pm 0.2$  minutes in NormTrol, followed by SKOV-3  $1.6 \pm 0.2$ , PC9 at  $1.8 \pm 0.1$ , PANC-1  $4.9 \pm 1.1$  while MIA PaCa-2s are least at  $7.6 \pm 1.4$  minutes. Amongst the hematological cells, U937 was the quickest and required  $9.8 \pm 1.6$  minutes to initiate TG while MM.1S was the slowest, taking  $16.2 \pm 2.8$  minutes (Table 1). The intra assay C of V of Lag times, ETP and the rest of the TG parameters were 4.78%, 3.53% and < 5% respectively, while the inter assay C of V of all the TG parameters were < 10%.

### *Clotting times and Thrombin generation*

To compare the TG parameters with another test of procoagulant activity, UMSCC81B cell lines at decreasing concentrations were selected for the prothrombin assay with a coagulometer as described above. When compared with TG parameters, CT for UMSCC81Bs had the highest correlation with the thrombin Peak ( $r^2 = -0.99663$ ,  $p = 0.0007$ ) Figure 2, followed by times-to-peak ( $r^2 = 0.94$ ,  $p = 0.0006$ ). It also correlated less strongly with other TG parameters with Pearson coefficient's of  $\leq 0.7$  but at lower or no significance (data not shown).

*Influence of coagulation factors on thrombin generation in cells from solid tumours*

TG parameters in NormTrol and coagulation factor VII-deficient plasma indicated significant differences (Table 1, Figure 3), but TG parameters in NormTrol and factor XII-deficient plasma were mostly insignificant. For example, AsPC-1 had a  $1.5 \pm 0.2$  minute Lag time in NormTrol and under factor XII-deficiency, the duration of which was prolonged to  $16.8 \pm 6.5$  minutes with factor VII absence. Interestingly, there was a smaller change or slowing down in hematological cells, such that absence of factor VII in platelet-free plasma apparently prolonged Lag times to a much lesser extent (Figure 3). An example is MM U266B, for which Lag times under normal plasma conditions was  $15.2 \pm 1.3$  minutes, but this time increased to  $19.3 \pm 3.4$  minutes under factor XII absence, and further increased to  $23.7 \pm 3.1$  minutes in factor VII absence.

Under coagulation factor VII-deficient plasma conditions, TG in the majority of cell lines and control was affected, with significant delay in initiation, volume and times to reach peak, and TG was not completed in all the MM cells in 60 minutes (Figure 4). For example, CFPAC-1 thrombin production reduced from 220 to 120 nM in factor VII-deficiency, a 45% reduction, while TG remained near 225nM in factor XII-deficiency. Conversely, for hematological cells only U937 retained the ability to complete its TG curve in 60 minutes when factor XII was absent, at a reduced volume and velocity (from  $17.8 \pm 1.1$  to  $39.2 \pm 6.4$  nM/min) while the others did not complete TG in the duration of experiment.

In all cell lines, times-to-peak and Lag times are the TG parameters that showed the highest number of significant changes under the conditions described, followed by Peak thrombin produced, Velocity index, and ETP (Table 1).

### *Influence of tissue factor on cell-induced thrombin generation in platelet-free plasma*

As a measure of the influence of cell TF expression on TG of cell lines, mean TF cell surface expression assessed by flow cytometry and plotted against average TG parameters using data generated at  $0.63 \times 10^6$  cells/ml. There was variability in TF expression as expected across the cell lines however; the solid cancer cell lines expressed higher TF values than hematological cells (Figure 5). While UMSCC81B expressed the highest TF amongst solid cancer cells overall, CFPAC-1 expressed the highest TF amongst PC cells and JLN3 the lowest of all cell lines. An inverse correlation was seen upon correlation of TF expression with TG parameters Lag time and TTP (Fig. 5). Upon  $\text{Log}_{10}/\text{Log}_{10}$  transformation of data with regression, a near linear relationship of these 2 TG parameters in NormTrol with TF expressed per  $10^5$  cells was displayed. Correlation with TG parameters obtained from factor VII-deficient conditions showed weak correlations of low significance ( $r^2 < 0.5$ ) and no relationship with factor XII-deficient conditions. Also, the thrombin generation parameters ETP, Vel.Index and peak height correlated weakly with TF cell surface expression ( $r^2=0.0548$ ,  $p < 0.01$ ,  $r^2=0.0548$ ,  $p < 0.01$  and  $r^2=0.0548$ ,  $p < 0.01$  respectively) (figures not shown).

### **Discussion**

Cancer cell lines exhibit TG in a cell concentration dependent manner. Of the TG parameters, lag time and time-to-peak highlighted the differences between solid and hematological tumours and were dependent on TF expression. Coagulation driven by solid tumour was also more TF-FVII dependent than for hematological cells, which was both factors VII and XII dependent, which suggests that there is a role for factor XII in the absence of factor VII in non-solid malignancies.

An ETP of 30% or higher has been linked to clinical situations where bleeding tendencies were absent or low, and therefore to the severity of observed bleeding[17]. In plasma, several studies suggest that the peak thrombin height and ETP are considered most important [18], but none has been conclusively determined for other substrates. Lag times reported here were defined as the time in which 20nM of thrombin is formed [9] and the importance of this initiation phase has been previously described in coagulation studies with limited factor V component[19]. Peak thrombin may reflect the ability of the cell lines to generate thrombin; however, it only correlated with a one-step clotting assay. This could mean that although the height of thrombin produced may reflect clotting time, it may not necessarily indicate the absolute capacity of the cell lines to generate thrombin.

Pancreatic cancer displays a TF-driven coagulation process when compared to MM that is not as dependent on TF-related coagulation [16, 20]. As to be expected, PC and other solid cancer cell lines exhibited a stronger overall TG profile in this study with larger volumes of thrombin produced. TG parameter differences characterize cancer cell lines, which may reflect how thrombin is formed *in vivo* and potential response to therapy. In this study, MM cells generally have slower lag times and may continuously generate thrombin for a longer period. A further distinction could also be made between the PC cell lines, where the fastest lag times and times-to-peak of those such as AsPC-1 and CFPAC-1 may be explained by increased cell surface mucin, unlike for PANC-1 and MIA PaCa-2 [21-23] which are slower to initiate TG. Although UMSCC81B has the highest TF cell surface expression this did not necessarily translate into the cell line with the highest total amount of thrombin produced, as only a little TF is needed to produce the initial thrombin necessary for amplification to the propagation phase of the clotting hemostatic cascade [24].

Compared to low TF control, factor VII exerted more influence than factor XII in both PC and MM. However, factor XII had a greater influence on the MM cells and almost none on PC.

Factor VII and factor XII are members of the extrinsic and intrinsic contact-activated coagulation pathways involved in thrombus formation[14]. They may also be involved in other functions such as inflammation and angiogenesis *in vivo* [25, 26]. Lack of both factors caused changes in TG parameters, which was consistent across cell lines. Solid tumour cells, such as PC express more TF, compared to hematological cell lines. TF-factor VII complex has been suggested to drive coagulation from a damaged vessel wall while factor XII may act at more distant locations [27] and may modulate stability of thrombus independent of thrombin generation[28]. TF-dependent pathways are known to utilise factor VII which activates the PAR-2 pathway and leads to a TF-FVII complex that activates the extrinsic clotting pathway [26, 29]. Although, TF-factor VII removal from the assay caused the most profound change in the TG kinetics, it is interesting to note that there was no complete abolishment in both cell groups, highlighting the possibility of an alternative pathway being utilized in these situations. Marchetti et al also reported a similar observation upon blocking of TF activity with anti-TF antibody during TG [13]. Moreover, our study shows that although TF cell expression correlates with some particular TG parameters, it does not singularly determine the level of thrombin produced.

Hematological cells under factor XII-deficient conditions behaved differently from solid cancer cells by generating less thrombin in a longer time with slower initiation. It could be suspected from this result that some level of inhibition of the contact pathway is lifted, such that though lower levels of thrombin height is reached, there is continuous thrombin production beyond 60 minutes. Although factor XII-deficiency is not associated with excessive bleeding its involvement in stabilising thrombus clot may contribute to increased risk of emboli formation [14]. It has also been suggested that some reduction in the levels of factor XII may result in higher cardiovascular risk and thrombotic complications[25, 30]. Also, though it can be bypassed by other coagulation cascade members, factor XII may still be essential and its importance comes to prominence in myeloma-related thrombosis in the

absence of other constituents[25], as seen here when TG persisted in smaller quantities after factor VII absence. Moreover, continuing TG may be responsible for persistent initiation of the clotting cascade leading to continuous clotting formation in clinical scenarios as suggested by Duchmein et al [18]. This study has indicated a possible large intrinsic pathway reserve for hematological cells and to a lesser extent solid cancer cells, and highlights differential contributions to TG by both the intrinsic and extrinsic coagulation pathways. This is supported by a previous study that has highlighted the possibility of a large intrinsic pathway reserve in human plasma samples of different origins[31].

The absence of factor XII may initiate an alternative TG pathway. For example a recent study found that in some solid cancers such as prostate and pancreatic cancer, TG might be triggered by polyphosphate exposure on their plasma membranes in a factor XII-dependent manner[27]. Therefore, the importance of the role of the intrinsic pathway may not be restricted to non-solid cancers alone. Furthermore, a switch from one factor-dependency to another might be possible in other cell lines and substrates.

## Conclusion

Cell from solid tumours exhibit greater thrombin generation potential and are faster to initiate coagulation than hematological cells. The use of Factor VII deficient plasma had a greater influence in thrombin generation in the solid tumour cells suggesting a greater reliance on the extrinsic, TF-driven pathway in these cells.

## Ethics statement

The use of commercially available cell lines such as those used in this study have been approved at a School level within the University of Hull.

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320           Conflict of interest

321           The Authors declare no conflict of interest.

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